PB82-154899

Aqueous Relaxation Reagents in Nitrogen-15 NMR

Florida State Univ., Tallahassee

Prepared for

Health Effects Research Lab.
Research Triangle Park, NC

20 Oct 79

U.S. Department of Commerce
National Technical Information Service
NTIS.

TECHNICAL REPORT DATA (Please read Instructions on the reverse before completing)		XX-81-066
1. REPORT NO. EPA-600/J-80-377	2. JOURNAL ARTICLE	3. RECIPIENT'S ACCESSION NO. PB82-154899
4. TITLE AND SUBTITLE Aqueous Relaxation Reagents in Nitrogen-15 NMR		5. REPORT DATE January 1982
		6. PERFORMING ORGANIZATION CODE 025109
7. AUTHOR(S) James J. Dechter and George C. Levy		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Chemistry The Florida State University Tallahassee, Florida 32306		10. PROGRAM ELEMENT NO. CCAE1A
		11. CONTRACT/GRANT NO. Grant No: R804916
12. SPONSORING AGENCY NAME AND ADDRESS Office of Research and Development (RTP,NC) Health Effects Research Laboratory US Environmental Protection Agency Research Triangle Park, NC 27711		13. TYPE OF REPORT AND PERIOD COVERED
		14. SPONSORING AGENCY CODE EPA-600/11

15. SUPPLEMENTARY NOTES

Published In: Journal of Magnetic Resonance 39: 207-215, 1980

16. ABSTRACT

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17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY NATIONAL TECHNICAL INFORMATION SERVICE U.S. DEPARTMENT OF COMMERCE SPRINGFIELD, VA 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
RELEASE TO PUBLIC	UNCLASSIFIED	11
	20. SECURITY CLASS (This page) UNCLASSIFIED	22. PRICE

Form 2220-1 (9-73)

EPA-600/J-80-377
Journal Article

JOURNAL OF MAGNETIC RESONANCE 39, 207–215 (1980)

Aqueous Relaxation Reagents in Nitrogen-15 NMR

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Received July 9, 1979; revised October 20, 1979

Electron–nuclear relaxation times (T_1^e's) for ^{15}N and ^{13}C in natural abundance are measured for a series of amines in aqueous solution using Gd(III) complexes of a series of polyaminocarboxylate ligands as paramagnetic relaxation reagents (PARRs). The PARRs are classified by their predominant mode of interaction with the amine substrates (i.e., specific or non specific). The specific PARRs are evaluated qualitatively as NMR spin labels through their selectivity as measured by ^{15}N T_1^e's toward subtrates of different Lewis base strength and through the degree of scalar line broadening induced in the substrate resonances. Additionally, the aqueous PARRs are compared with better characterized nonaqueous PARRs, $Cr(acac)_3$ and $Gd(dpm)_3$. It is concluded that the diethylenetriaminepentaacetic acid complex, $Gd(DTPA)^{2-}$, is a satisfactory specific PARR and NMR spin label. The triethylenetetraaminehexaacetic acid complex, $Gd(TTHA)^{3-}$, is shown to be a useful non specific PARR although it is a charged complex; the presence of charged groups in substrates results in weak spin label effects. Finally, the behaviour of these PARRs is contrasted with that of hydrated transition metal and lanthanide ions.

INTRODUCTION

The NMR literature is replete with studies concerning the use and evaluation in organic solvents of paramagnetic relaxation reagents (PARRs) for carbon-13 (*1, 2*), silicon-29 (*3*), and nitrogen-15 (4, 5). Aside from applications involving bare paramagnetic transition metal ions and lanthanide ions bound to proteins and other biomolecules, there are only two references in the literature where PARRs suitable for aqueous solution are applied to carbon-13 NMR (*6, 7*). The use of the solvated lanthanide ions is of limited interest for ^{15}N NMR since the hydroxide precipitates above pH 6. Additionally, there have been a few studies primarily concerned with the evaluation or use of aqueous shift reagents which have also used the relaxation effect of Gd^{3+} complexes (*8–12*). This paper is concerned with the qualitative evaluation of a class of PARRs for nitrogen-15 in aqueous solution, where alkaline conditions and the presence of strong Lewis bases severely complicate the use of PARRs. It is to be emphasized that the success of a PARR in carbon-13 or proton NMR does not assure its success as a nitrogen-15 PARR (vide infra).

There are two classes of PARRs and they can be characterized by the predominant mechanism of relaxation inducement. *Non specific* PARRs effect relaxation via a translational diffusion or outer-sphere mechanism. This type of PARR is used to

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0022-2364/80/080207-09$02.00/0

Printed in Great Britain

shorten the spin–lattice relaxation time, facilitating data acquisition, or to eliminate nuclear Overhauser effects (NOEs), allowing quantitative analysis of the signal areas. For ^{15}N (and other nuclides with negative magnetic moments) there is a need to quench the NOE when a partial dipolar relaxation contribution results in reduced or nulled signals. The best PARRs for this type of application in organic solvents have been the Cr(III) complexes of acetylacetone (acac) (*1, 2, 4, 5, 13*), or better, of dipivaloylmethane (dpm) (*13a*). The success of these PARRs arises from the nonlability and the coordinative saturation of octahedral Cr(III) complexes.

The second type of PARR is termed *specific* since an actual complex is formed with the substrate. Spin–lattice relaxation is induced by an intramolecular electron–nuclear dipolar interaction which has a sixth-power dependence on the separation distance and also depends on the overall tumbling rate of the PARR–substrate complex. This type of PARR enables one to do NMR spin labeling (*5*), exploiting the sensitive distance dependence of the relaxation rate. For organic solvent systems, $Gd(dpm)_3$ has been the most widely used specific PARR (*5, 14*). The ability of the lanthanides to expand their coordination spheres to accommodate higher coordination numbers is responsible for their mode of interaction in the organic solvents.

Several PARRs suitable for aqueous systems and which might behave non specifically in the presence of strong Lewis bases are mentioned in the literature. Bose *et al.* have described the use of the ethylenediamine (en) complex of Cr(III) (*6*). Since this is positively charged, it was found to act as a specific PARR when anionic groups are present in the substrate. Much weaker coordination was apparent with a nonanionic Lewis base. Lettvin and Sherry reported the use of the triethylenetetraaminehexaacetic acid (TTHA) complex of Gd(III) (*7*), which they found to be well behaved over the pH range 3–12. They noticed a very slight specific interaction with anionic carboxyl groups, similar to, although much weaker than, the interaction observed with $Cr(en)_3^{3+}$.

There have been several reports of the use of $Gd(EDTA)^-$ as a broadening probe in ^1H NMR structural studies (*8–11*). Elgavish and Reuben have evaluated the behavior of $Gd(EDTA)^-$ over a wide pH range using acetate and a series of ammonium ions with increasing methyl substitution as substrates (*10, 11*). They found the useful pH range to be 6 to 10. In all of these investigations using $Gd(EDTA)^-$, proton line broadening has been interpreted as arising from a dipolar mechanism and distances were determined. Similar ^1H substrate line broadening was found using a dipicolinic acid complex of Gd^{3+}, $Gd(DPA)_3^{3-}$ (*12*). Espersen and Martin have demonstrated for Cu^{2+} and Mn^{2+} that induced line broadenings are predominantly due to a scalar T_2 mechanism (*15*) and therefore distance information cannot be obtained. It has been shown that $Gd(fod)_3$ induces significant contact shifts (*14*). Therefore, in any evaluation of PARRs for ^{15}N an important consideration is the determination of the relaxation mechanism through the determination of T_1^c/T_2^c for the nitrogen resonance.

Based on these reports, we have initiated an evaluation of the polyaminocarboxylate complexes of Gd^{3+} as aqueous PARRs for nitrogen-15. We have also included the nitrilotriacetic acid (NTA) and the diethylenetriaminepentaacetic acid (DTPA) complexes of Gd^{3+}. Comparison is made with results using $Cr(en)_3^{3+}$. As substrates, we have chosen a series of cyclic amines: pyridine, imidazole, and

pyrrolidine. The range of Lewis basicity as judged by the pK_a values of 5.26, 6.95, and 11.3 (*16*) provides an excellent test of specific versus nonspecific behavior. The ring allows a second test of this behavior through the comparison of T_1^e's for all nuclei in the ring. T_1^e's, unlike linewidths, are not significantly affected by scalar contributions to relaxation (*5*) and will contain distance information. The comparison of T_1^e with T_2^e determined from line broadenings provides an evaluation of nondipolar contributions to line broadening.

Finally, because of the high concentration of amine substrate necessary for facile natural abundance ^{15}N T_1 studies, and the known sensitivity of ^{15}N to paramagnetic impurities (*17*), it was not considered desirable to introduce the large quantities of acid or base necessary for a pH profile. Based on the ^1H studies of Elgavish and Reuben (*10, 11*) and the known pK_a's of the polyaminocarboxylate ligands (*18*), as long as the solution pH is near the substrate pK_a, optimum relaxation effects of the Gd(III) PARR should be achieved. Determination of the ^{15}N pH relaxation profile will require the use of ^{15}N-enriched substrates.

EXPERIMENTAL

The liquid amines used as substrates were commercially obtained and purified by standard techniques (*16*). Water was distilled and deionized. The polyaminocarboxylic acids were commercially obtained and used with no further purification. Gd(III) complexes were prepared as stock solutions (except for the TTHA complex) by adding stoichiometric amounts of $Gd(NO_3)_3 \cdot 5H_2O$ and ligand and adjusting the pH to achieve dissolution (usually about pH 10). $Cr(en)_3Cl_3 \cdot 3H_2O$ was prepared by a standard procedure (*19*). Viscosities were measured using Cannon–Manning semimicro viscometers and were found to be 1.61 cP for 6 *M* imidazole, 3.03 cP for 6 *M* pyrrolidine and 1.77 cP for 6 *M* pyridine solutions. The pH's of the solutions were found to be 10.6, 12.6, and 11.7, respectively, after addition of $Gd(DTPA)^{2-}$.

Nitrogen-15 spectra were obtained at 27.4 MHz and carbon-13 spectra at 67.9 MHz on a Bruker HX-270 spectrometer. Measurements were made using 15-mm sample tubes with a coaxial 5-mm tube containing acetone-d_6 as the lock material. Spin–lattice relaxation times were determined using the fast inversion–recovery technique (FIRFT) (*20*) and were calculated using an on-line, three-parameter, nonlinear least-squares fitting procedure (*21*).

THEORY

The relaxation mechanism for specific PARRs arises from the spin–dipolar interaction of the electronic and nuclear spins. The form of this interaction is given by Solomon (*22*) and Bloembergen (*23*) and is shown in Eq. [1].

$$\frac{1}{T_1 e} = \frac{2S(S+1)\gamma_I^2 \gamma_S^2 \hbar^2}{15 r^6} f(\tau_c). \qquad [1]$$

This equation applies when there is a complex formed between the substrate and PARR. S is the electronic spin, γ_I and γ_S are the magnetogyric ratios of spins I and S,

r is the distance between the two spins and $f(\tau_c)$ represents a function of the correlation time for this dipolar process. Since the electron spin relaxation times for Cr(III) and Gd(III) are relatively long (*24*) and since no evidence exists for an exchange contribution to the correlation time, it can be assumed that τ_c is dominated by τ_r, the rotational reorientation time of the substrate–PARR complex. Under the condition $\omega_I^2\tau_c^2 \ll 1$ and $1 \ll \omega_S^2\tau_c^2$ and assuming the microviscosity-corrected Stokes–Einstein model to describe the solution reorientation of the complex (*25*), one obtains

$$\frac{1}{T_1^e} = \frac{2S(S+1)\gamma_I^2\gamma_S^2\hbar^2}{5r^6}\left(\frac{\eta V_m}{f_r kT}\right). \qquad [2]$$

Here, η is the macroscopic viscosity, V_m is the molecular volume of the complex, and f_r is the microviscosity factor.

The translational diffusion mechanism does not require the formation of a complex between the PARR and the substrate. Although a more detailed theory exists (*26*), the simpler formulation by Abragam (*27*) is given in Eq. [3].

$$\frac{1}{T_1^e} = \frac{16\pi}{15} N_S S(S+1)\gamma_I^2\gamma_S^2\hbar^2 \frac{\eta}{kT}. \qquad [3]$$

N_S is the number of paramagnetic species per unit volume of the solution. The other terms have the previously described meanings. Equation [3] applies strictly for similar-sized spherical species; however, it has been shown to apply in a qualitative sense for systems similar to those described here (*5*).

For the purpose of the subsequent discussion, there are several important aspects of these equations. First, both Eqs. [2] and [3] show a dependence of the relaxation rate on γ_I^2 and η. This forms the basis for normalization of T_1^e data to a common magnetogyric ratio and viscosity. In this manner, T_1^e for different nuclides in different solutions may be directly compared. Second, both equations exhibit a dependence of the relaxation rate on $S(S+1)$. Gd^{3+} with seven unpaired electrons will be 4.7 times more effective a PARR than Cr^{3+} with only three unpaired electrons, as long as the interaction mode (e.g., specific vs nonspecific) is the same and the concentrations are the same. Finally, a wide variety of weak interactions are termed outer-sphere. Any specificity in T_1^e due to these weak interactions is usually small because of the large distances involved. For the purposes of the qualitative

TABLE 1

NITROGEN-15 T_1^e's (cP-sec) FOR 2×10^{-3} *M* $Gd(NO_3)_3$ WITH SELECTED LIGANDS

Substrate[a]	TTHA(1:1)[b]	DTPA(1:1)	EDTA(1:1)	NTA(2:1)	DPA(3:1)	$Cr(en)_3Cl_3$[c]
Imidazole	4.4	3.3	1.1	6.1	4.7	5.0
Pyrrolidine	3.8	7.8	11	11	8.7	7.4
Pyridine	4.5	21	8.0	19	24	8.2

[a] 6 *M* aqueous solution.
[b] 0.01 *M* $Gd(NO_3)_3$.
[c] 0.05 *M* $Cr(en)_3Cl_3$.

discussion which follows, the PARRs will be classified by their predominant mode of interaction. A quantitative separation of the two mechanisms (*5*) will not be attempted here.

The electron nuclear relaxation rate is experimentally obtained as the difference between the relaxation rates measured in the presence and absence of the PARR as shown in Eq. [4].

$$\frac{1}{T_1^e} = \frac{1}{T_1^{\text{Para}}} - \frac{1}{T_1^{\text{Dia}}} . \qquad [4]$$

RESULTS AND DISCUSSION

Table 1 presents the *normalized* nitrogen-15 T_1^e's (i.e., corrected for viscosity) induced by the Gd^{3+} complexes of a series of polyaminocarboxylic acids on three cyclic amines of different basicities. The stoichiometric ratio of ligand to Gd^{3+} is indicated in parentheses for each ligand. With the exception of the TTHA complex, the concentration of Gd^{3+} was 2×10^{-3} *M*. For TTHA, complexation of the substrate was so weak that a fivefold increase in the concentration of the PARR was necessary, indicative of a different mode of interaction. 0.05 *M* $Cr(en)_3^{3+}$ is included for comparison. The aqueous solutions are 6 *M* in substrate. The T_1^e's are corrected for viscosity and are reported in units of centipoise-seconds, essentially normalizing all solutions to a viscosity of 1.0 cP. In all cases, the pH was sufficiently high that all ligand carboxyl groups were fully ionized (*18*).

It is possible to qualitatively determine the predominant mechanism of interaction by comparing the ^{15}N T_1^e's for the same PARR as a function of substrate (i.e., reading down the column in Table 1). A nonspecific PARR is identifiable by a constant T_1^e; a specific PARR is identifiable by a decrease in T_1^e with increasing complexing ability of the substrate. Table 1 indicates that all the PARRs undergo specific interactions with the substrate to some degree with the exception of the TTHA complex. Note also that the concentration of the PARR for the TTHA case is five times greater than for the other gadolinium chelates—a condition which should result in a fivefold reduction in the T_1^e for an identical mode of interaction. $Cr(en)_3^{3+}$ was also used at a higher concentration since it was anticipated to be an outer-sphere PARR as discussed above, but Table 1 indicates some complexation which may be due to degradation of the PARR in the presence of strong Lewis bases.

The effect of the number of unpaired spins for Gd^{3+} and Cr^{3+} should make Gd^{3+} about 4.7 times as efficient a PARR as Cr^{3+}—again, for a constant mode of interaction. This factor is qualitatively observed by comparing the T_1^e's in the presence of the Gd^{3+}–TTHA complex which is 0.01 *M*, with the T_1^e's in the presence of $Cr(en)_3^{3+}$, where the PARR is 0.05 *M*. The fivefold concentration factor approximately cancels the fivefold spin factor and the T_1^e's are similar in magnitude.

The Gd^{3+} complexes of DTPA and DPA appear to be the most sensitive reagents to substrate complexing ability or to substrate basicity (i.e., they exhibit the greatest selectivity). A striking feature of Table 1 is that imidazole, for all PARRs except $Gd(TTHA)^{3-}$ has the shortest T_1^e whereas pyrrolidine is much more basic and on the basis of pK_a alone would be expected to have the shortest T_1^e. The pK_a for

pyrrolidine is 11.3, while for imidazole the pK_a is 6.95. Metal complexation of imidazole is known to increase the acidity of the pyrrole-type nitrogen (*28*). Possibly the ability of this acidic proton to hydrogen bond to the polyaminocarboxylic acid ligand might cause an increased complexing ability for imidazole with these PARRs. This selectivity behavior could be further investigated through a pH-dependent study of ^{15}N-enriched material at lower concentrations.

As was mentioned previously, T_1^e has not been shown to be significantly influenced by scalar interaction of the nucleus with the unpaired electronic spins of the PARRs (*5*). However, T_2^e can commonly be dominated by this electron–nuclear relaxation mechanism (*15*). An important practical consideration in the evaluation of a PARR is its effect on the substrate linewidth, since minimum scalar broadening is desired. Table 2 summarizes the degree of nitrogen-15 line broadening induced in the substrate nitrogens. For the Gd^{3+} complexes of TTHA, DPA, and DTPA there is only slight or insignificant line broadening. NTA induces a higher degree of line broadening while EDTA and $Cr(en)_3{}^{3+}$ can cause enormous line broadening. The broadening effect of $GD(EDTA)^-$ has previously been observed (*8–11*) although it was incorrectly interpreted as being of dipolar origin.

The dipicolinic acid complex would appear to be acceptable as a specific PARR on the basis of T_1^e selectivity and limited line broadening, but solutions containing this PARR precipitate the metal hydroxide upon standing and thus show no long-term solution stability. By contrast, solutions of $Gd(DTPA)^{-2}$ are essentially stable with little change in the T_1^e for periods of at least several days.

It is worth noting that Mn^{2+}, which has been used extensively as an NMR line broadening and relaxation probe for biomolecules, is not suitable for general use as an aqueous ^{15}N PARR. Aqueous solutions of pyrrolidine are sufficiently alkaline to precipitate $Mn(OH)_2$. Mn^{2+} is more stable in less alkaline solutions such as 6 *M* pyridine; however, at a concentration sufficient to produce a normalized T_1^e of 7.8 cP sec (1×10^{-4} *M* Mn^{2+}), 19 Hz of scalar line broadening is observed. The scalar broadening effect of Mn^{2+} and Cu^{2+} on ^{13}C resonance lines has been described (*15*). Aqueous Gd^{3+} ion without an associated ligand is not useful since the hydrated ion is ineffectual as a PARR and is easily precipitated as the insoluble hydroxide at relatively low pH's. At concentrations of 2×10^{-3} *M*, ^{15}N T_1^e's are still relatively long (ca. 70 cP sec).

TABLE 2

NITROGEN-15 LINE BROADENING (Hz) UPON ADDITION OF 2×10^{-3} *M* OF SELECTED PARRs

Substrate[a]	TTHA(1:1)[b]	DTPA(1:1)	EDTA(1:1)	NTA(2:1)	DPA(3:1)	$Cr(en)_3Cl_3$[c]
Imidazole	—	—	76	10	2	75
Pyrrolidine	—	5	5	8	2	32
Pyridine	—	3	17	3	6	2

[a] 6 *M* aqueous solution.
[b] 0.01 *M* $Gd(NO_3)_3$.
[c] 0.05 *M* $Cr(en)_3Cl_3$.

TABLE 3

COMPARISON OF NORMALIZED CARBON/NITROGEN T_1^e RATIOS FOR AQUEOUS AND NONAQUEOUS PARRS

	$Gd(dpm)_3$ [a]		$Gd(DTPA)^{2-}$		$Cr(acac)_3$ [a]		$Gd(TTHA)^{3-}$	
Substrate	C_1/N	C_2/N	C_1/N	C_2/N	C_1/N	C_2/N	C_1/N	C_2/N
Imidazole	2.7	5.7	2.5	3.3	1.2	1.3	1.7	2.0
Pyrrolidine	9.1	17	2.6	4.1	1.1	1.3	1.4	1.9
Pyridine	2.2	6.2	1.5	1.8	1.0	0.9	1.0	1.5

[a] Nonaqueous PARR, T_1^e ratios calculated from Ref. (*5*).

Based on the results of Tables 1 and 2, $Gd(TTHA)^{3-}$ appears to be a promising outer-sphere or nonspecific PARR while $Gd(DTPA)^{-2}$ seems promising as a specific PARR. One way to further evaluate these PARRs is to compare their normalized ^{13}C and ^{15}N T_1^e's with those of well-characterized nonaqueous PARRs such as $Cr(acac)_3$ and $Gd(dpm)_3$. Such a comparison is presented in Table 3.

Since the substrates are rigid molecules, and any specific interaction with the PARR is through the nitrogen lone pair, complexation is characterized by an observed increase in the normalized T_1^e's from the nitrogen to the far end of the molecule. For nitrogen, the normalized T_1^e's are those given in Table 1 (i.e., normalized to 1.0 cP). Carbon-13 T_1^e's are additionally normalized to the magnetogyric ratio of nitrogen (i.e., multiplied by $(\gamma_C/\gamma_N)^2$). These *normalized* carbon and nitrogen T_1^e's may be directly compared. Viscosity normalization facilitates comparison between different solutions as well (see Table 1). In Table 3, the *ratios* of normalized T_1^e's, C_1/N and C_2/N, are presented since these ratios should be independent of concentration, merely reflecting the degree of complexation with the PARR. Totally isotropic translational diffusion would be associated with a ratio of 1.0 for both C_1/N and C_2/N as is seen for the system of pyridine and $Cr(acac)_3$.

Table 3 compares the ratios of *normalized* T_1^e's for a well-characterized nonaqueous specific PARR, $Gd(dpm)_3$, and for $Gd(DTPA)^{-2}$. T_1^e's are also presented for a well-characterized nonaqueous nonspecific PARR, $Cr(acac)_3$, and for $Gd(TTHA)^{-3}$. There are two striking features in the comparison of $Gd(dpm)_3$ and $Gd(DTPA)^{2-}$. First, since hydrogen bonding to the ligand by imidazole is not possible with $Gd(dpm)_3$, the C_1/N ratio of the substrates is seen to correlate with substrate basicity. Second, the C_1/N ratios of the substrates with $Gd(dpm)_3$ as a PARR increase by a factor of 4 from pyridine to pyrrolidine while with $Gd(DTPA)^{-2}$ as a PARR this increase is only a factor of 1.7. Thus $Gd(DTPA)^{2-}$ complexation is not as sensitive to subtle changes in substrate basicity as is that of $Gd(dpm)_3$.

$Cr(acac)_3$ is seen to be well behaved as a nonspecific PARR for these substrates although hydrogen bonding to acidic substrate protons has been shown to occur (*13*). While $Gd(TTHA)^{3-}$ is not as well behaved, when hydrogen bonding to the ligand or electrostatic interactions with an anionic group are absent this PARR is predominantly outer sphere in its mode of action. Certainly in terms of the nitrogen T_1^e's in Table 1, $Gd(TTHA)^{3-}$ appears to be satisfactory as a nonspecific PARR.

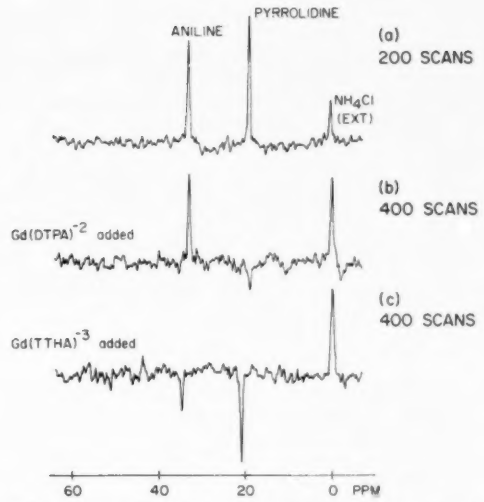

FIG. 1. Nitrogen-15 NMR spectra, 27.4 MHz, of an aqueous mixture that is 3 *M* in aniline and 3 *M* in pyrrolidine. A 45° pulse width was used with a 15-sec pulse repetition rate. (a) Diamagnetic solution, ^{15}N T_1^{dd}'s are comparable (ca. 45 cP-sec) with essentially full NOEs; (b) 2×10^{-3} *M* $Gd(DTPA)^{2-}$; (c) 0.02 *M* $Gd(TTHA)^{3-}$. Spectra are phased such that the equilibrium diamagnetic spectrum with full NOE is phased with all resonances positive.

As a test of the spin label ability of the specific PARR $Gd(DTPA)^{2-}$, an aqueous solution 3 *M* in pyrrolidine and 3 *M* in aniline was prepared. As we discussed previously (*5*), under conditions of comparable dipolar T_1's (T_1^{dd}) and comparable NOEs, the quenching of the NOE may be used as a measure of spin label ability. Here, advantage is taken of the negative magnetic moment of nitrogen-15. Figure 1a shows the diamagnetic nitrogen-15 NMR spectrum of the above mixture. The high field resonance is an external $^{15}NH_4Cl$ chemical shift reference. Upon addition of 2×10^{-3} *M* $Gd(DTPA)^{2-}$ the NOE of the basic pyrrolidine nitrogen is sufficiently quenched to phase invert the signal. The signal of the much less basic aniline nitrogen ($pK_a = 4.63$) is affected far less as is shown in Fig. 1b. When 0.02 *M* $Gd(TTHA)^{3-}$ is used as a PARR, both NOEs are similarly affected and both resonances are phase inverted as is shown in Figure 1c. The weak specificity of $Gd(TTHA)^{3-}$ is illustrated by the intensity difference between the aniline and pyrrolidine resonances, indicating a slightly more efficient quenching of the pyrrolidine nitrogen NOE.

CONCLUSIONS

Of the polyaminocarboxylate complexes of Gd(III) investigated here, it is possible to single out the diethylenetriaminepentaacetic acid complex, $Gd(DTPA)^{2-}$, and the triethylenetetraaminehexaacetic acid complex, $Gd(TTHA)^{3-}$, as suitable for use as aqueous PARRs in nitrogen-15 NMR. Both are stable in strongly alkaline media and in the presence of strong Lewis bases. From the analysis of nitrogen T_1^e's for amine substrates covering a reasonable range of pK_a's it is concluded that $Gd(DTPA)^{2-}$ is a

satisfactory specific PARR and is adequate for NMR spin labeling, while $Gd(TTHA)^{3-}$ is a satisfactory nonspecific PARR. It should be possible, now that the proper relaxation behavior has been determined for these complexes, to investigate ^{15}N-enriched substrates at lower concentrations. By investigating the pH behavior of relaxation, it will be possible to identify the interacting species.

ACKNOWLEDGMENTS

Acknowledgment is made to the donors of the Petroleum Research Fund administered by the American Chemical Society for partial support of this research and to the U.S. Environmental Protection Agency for Grant R804916.

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